Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part C 145 (2007) 350–362
CBP
www.elsevier.com/locate/cbpc

The identification of heat shock protein genes in goldfish (*Carassius auratus*) and their expression in a complex environment in Gaobeidian Lake, Beijing, China

Jianshe Wang, Yanhong Wei, Xuemei Li, Hong Cao, Muqi Xu, Jiayin Dai *

Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, 25 Beisihuan Xilu, Beijing 100080, PR China

Received 14 November 2006; received in revised form 7 January 2007; accepted 11 January 2007
Available online 22 February 2007

Abstract

The enhanced expression of heat shock proteins (HSPs) can be detected in response to high temperatures, as well as to many kinds of stressors, including pollutants. Partial cDNA sequences encoding HSP30, HSP70, HSP90 beta, and heat shock cognate (HSC) 70, and full-length cDNA sequences encoding HSP27, HSP47 and HSP60 were cloned from goldfish (*Carassius auratus*). The expression of these genes was investigated in goldfish inhabiting Gaobeidian Lake in Beijing, China. The water of this lake is moderately polluted and has a higher temperature due to the water being used as a coolant in the nearby thermal power plant. All HSP sequences tested were highly conserved compared with their corresponding genes in other species. A significant up-regulation in HSP30 and HSP70 transcripts was exhibited in goldfish collected in winter in Gaobeidian Lake. The up-regulation in HSP27 and HSP90 beta transcript, as well as HSP30, was observed on the day of collection in summer. The up-regulation of these HSPs suggested that fish under these specific environmental conditions were experiencing a complex stress process. The expression of HSP30 was found to be more prominent among the fishes in Gaobeidian Lake than at the cleaner reference site (Huairou Reservoir). In the latter case, the HSP30 expression was almost non-detectable, suggesting the possibility of using it as a biomarker for complex environmental pollution.

Keywords: Complex stress; Gene sequence; Goldfish; Heat shock protein

1. Introduction

When exposed to a variety of stresses such as extremes of temperature (Misra et al., 1989; Wagner et al., 1999), anoxia (Gamperl et al., 1998), oxidative stress (Broome et al., 2006), bacterial infections (Mayr et al., 2000), and various toxic substances (Airaksinen et al., 2003a,b), organisms synthesize a family of proteins called heat shock proteins (HSPs). HSPs have been highly conserved throughout evolution at the levels of gene sequence, genomic organization, gene transcription, and protein structure and function (Parsell and Lindquist, 1993). HSPs can be classified into several major families according to their molecular weight, e.g., HSP100, HSP90, HSP70, HSP60, and small heat shock proteins (sHSPs), etc. Serving as molecular chaperones, HSPs play a central role in cell protection by preventing protein aggregation and promoting disaggregation, proper refolding, and degradation of damaged polypeptides (Beckmann et al., 1990; Chiang et al., 1989; Basu et al., 2002).

Subjected to daily and seasonal temperature fluctuations, fishes are ideal model organisms for studying the heat shock response. The HSP family genes in zebrafish have been cloned (Mao et al., 2005) and their expressions have been examined under heat and pollutant stresses in the laboratory as well as in the field (Hallare et al., 2004, 2005; Airaksinen et al., 2003a,b; Murtha and Keller, 2003). However, few studies have been performed on goldfish (Kondo et al., 2004; Kagawa, 2004), one of the most common species in freshwater lakes in China.

The present study has focused on the cloning and sequencing of the gene products coding for goldfish HSPs, and also investigated the effect of the complex environmental stresses in Gaobeidian Lake on the expression of HSPs in goldfish.

* Corresponding author. Tel.: +86 10 62634802; fax: +86 10 62565689.
E-mail address: daijy@ioz.ac.cn (J. Dai).

1532-0456/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2007.01.018

2. Materials and methods

2.1. Study area

Gaobeidian Lake has a catchment area of some 0.15 km^2. Its water source is mainly the effluent of the Gaobeidian Wastewater Treatment Plant. Water in Gaobeidian Lake is used as a coolant by the nearby Beijing Guohua Thermal Power Plant, and then returned to the aquatic environment at a higher temperature than it was originally. Water temperatures in the lake are between 12 and 41 °C, corresponding to seasonal changes, which is some 5 to 10 °C higher than the ambient temperature. The water in Gaobeidian Lake discharges into Tonghui River and finally into the sea (Fig. 1). Some physicochemical water quality data in Gaobeidian Lake were as follows: dissolved oxygen: 3.1 ± 0.6 mg/L; suspended solids: 16.0 ± 3.4 mg/L; total phosphorus: 2.3 ± 0.7 mg/L; total nitrogen: 27.8 ± 4.4 mg/L; chemical oxygen demand: 46.3 ± 6.3 mg/L; temperature: 29.7 ± 10.6 °C; and pH 7.7 ± 0.1, indicating that the lake water is moderately polluted. Huairou Reservoir, dissolved oxygen: 6.1 ± 0.2 mg/L; total phosphorus: 0.013 ± 0.005 mg/L; total nitrogen: 0.60 ± 0.09 mg/L; chemical oxygen demand: 3.0 ± 0.6 mg/L; a drinking water source to the north of Beijing, was used as the reference site.

2.2. Experimental animals

Goldfish (body mass: 50.4 ± 4.5 g, body length: 11.9 ± 0.6 cm) were caught from Gaobeidian Lake and Huairou Reservoir on November 23, 2005 and May 16, 2006, respectively. The temperatures for the days of collection at the Gaobeidian Lake and Huairou Reservoir were 35 °C and 22 °C, 20 °C and 4 °C, respectively. Fish were killed by a sharp blow to the head followed by severance of the spinal cord, and dissected on ice. All the tissues were snap-frozen in liquid nitrogen, followed by storage at −80 °C.

2.3. Total RNA extraction and reverse transcription reaction

Total RNA was isolated from each tissue sample using Trizol (Invitrogen, USA) and treated with RNase-free DNase I (Qiagen, USA) to remove any remaining genomic DNA, according to the manufacturers' instructions. Reverse transcription was achieved using oligo $(dT)_{15}$ primer (Promega, USA) and M-MuLV reverse transcriptase (New England Biolabs, UK), according to the supplier's instructions. Conditions for reverse transcription were as follows: 60 min at 42 °C, followed by 5 min at 98 °C.

2.4. PCR and sequencing

Goldfish HSP30, HSP70, and HSC70 were amplified using primers designed based on sequences in GenBank (accession nos. AB177389, AB092839, and AB092840, respectively). Cross-species primers for HSP27, HSP47, HSP60 and HSP90 beta were designed based on consensus sequences in their zebrafish orthologs available in GenBank (accession no. BC097148, U31079, BC044557 and AF068772, respectively) (Table 1). PCR was performed using 1U of VentR ® DNA Polymerase or Taq DNA Polymerase (New England Biolabs, UK) at a final concentration of 1 × PCR buffer as formulated by New England Biolabs; 250 μM of dNTP; and 0.5 μM of each primer, set in a total volume of 20 μL. PCR conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 to 80 s (dependent on the size of the amplified fragment), with a final extension period of 72 °C for 10 min. To confirm the obtained sequence, PCR products were run on a 1.2% agarose gel containing ethidium bromide and

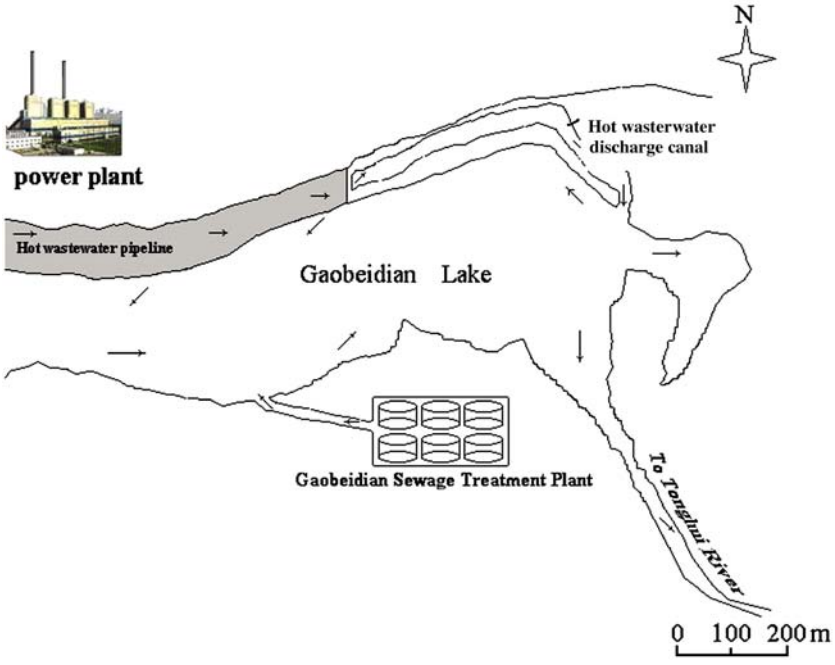

Fig. 1. Map of Gaobeidian Lake, Beijing, China. The locations of the sewage treatment plant, hot wastewater discharge canal, and Tonghui River are shown.

Table 1
List of primers for RT-PCR and their sequences

Gene	Primer sequence (5′–3′)	Product size (bp)
HSP27 forward primer	CTTTGGGATGCCACACTTCT	
HSP27 reverse primer	ATCTGGCGTCTGCTTGATCT	226
HSP30 forward primer	ACGCCACCAGAGAGACAACT	
HSP30 reverse primer	TGACGGCTGATTTACTGGTG	541
HSP47 forward primer	CACTGGGATGAGAAGTTCCA	
HSP47 reverse primer	AAGGAAAATGAAGGGATGGTC	539
HSP60 forward primer	CGGAAGATGTGGATGGAGA	
HSP60 reverse primer	GACGGCCACTCCATCTG	386
HSP70 forward primer	ATCCTGACCATTGAAGACGG	
HSP70 reverse primer	TGTTCAGTTCTCTGCCGTTG	457
HSC70 forward primer	CAATGAACCAACTGCTGCTG	
HSC70 reverse primer	CATGCGCTCAATGTCCTC	1036
HSP90 beta forward primer	ACCAAACACAACGATGACGA	
HSP90 beta reverse primer	CCGATGAACTGGGAGTGTTT	189
Beta-actin forward primer	GGCCTCCCTGTCTATCTTCC	
Beta-actin reverse primer	TTGAGAGGTTTGGGTTGGTC	156

the corresponding fragments were cut and purified using a QIAquick Gel Extraction Kit (Qiagen, USA). The corresponding fragments were then inserted into a pGEM-T vector (Promega, USA). Plasmids were purified from transformed DH5 alpha competent cells and sequenced on a commercial ABI 3730 capillary sequencer.

2.5. Rapid amplification of cDNA ends (RACE)

To obtain complete goldfish HSP27, HSP47 and HSP60 sequences, 5′- and 3′-RACE analyses were performed using the BD SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, USA), according to the manufacturer's protocol. The gene specific primers (GSPs) in Table 2 were designed and synthesized according to the initial partial cDNA sequence obtained from goldfish. PCR was amplified for 32 cycles with the following parameters: 94 °C for 1 min, 65 °C for 30 s, and 72 °C for 3 min. The amplified fragments were subcloned into a pGEM-T vector and sequenced.

2.6. Database and sequence analyses

The predictions for the amino acid sequences of goldfish HSP27, HSP47, and HSP60 were carried out using the open reading frame (ORF) finder programs in NCBI. Database search using the BLASTN and BLASTP programs was carried out to test the degree of nucleotide and amino acid sequence homology with other HSP sequences from vertebrates. Sequence alignments with their orthologs in other species were performed with the program clustalW in EBI.

2.7. Spatial expression of goldfish HSP27, HSP47, and HSP60 mRNAs in Huairou Reservoir

To detect the spatial expression of HSP27, HSP47, and HSP60 mRNA, total RNA was isolated from various tissues including brain, heart, skeletal muscle, kidney, gill, and liver in goldfish from Huairou Reservoir, treated with DNase I, and reverse-transcripted to cDNA as described in 2.3. The expression of HSP27, HSP47, and HSP60 were analyzed by semi-quantitative RT-PCR using the primers listed in Table 1. The transcripts of beta-actin were used as an internal control. The PCR products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining.

2.8. Alteration in HSP transcription levels in Gaobeidian Lake

To examine the effect of environmental stress in Gaobeidian Lake on the mRNA expression of HSPs, the transcripts of goldfish HSPs both in Gaobeidian Lake and at the reference site were quantified by semi-quantitative RT-PCR (samples collected on November 23, 2005) and real-time quantitative PCR (samples collected on May 16, 2006), respectively. Hepatic tissue samples from 20 fish were random pooled to three groups (n=7, 7, 6). RNA isolation and reverse transcription were performed as described in 2.3, and the amount of each HSP was normalized to the abundance of beta-actin. To test the RNA samples for DNA contamination, cDNA synthesis and subsequent amplification reactions were also performed in the absence of reverse transcriptase.

When semi-quantitative PCRs were done, different cycles were performed and band densities of the resulting products were measured using Bandleader Software. A cycle number within the exponential phase of the amplification curve was chosen for quantifying the expression of each gene in subsequent experiments.

Quantitative RT-PCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen, USA) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA) in a 50 μL total reaction volume including 25 μL of 2×QuantiTect SYBR Green PCR master mix, 2 μL cDNA template, and 0.2 μM each of target specific primers. The primers for HSP27, HSP60, HSP70, and HSP90 beta listed in Table 1 were used, and new primers for HSP30, HSP47, HSP60, and HSC70 were designed to amplify a 150–250 bp fragment of each gene. The sequences of the primer pairs were as follows: HSP30: forward primer, 5′-ATGTTGAGCCTGCATGGATT-3′; reverse primer, 5′-AAGTTTCTCCAGCTGCTCCA-3′. HSP47: forward primer, 5′-GGCATTTATGGCCTTTTTGA-3′; reverse primer, 5′-CAACTGCCCTCTGTTCCATT-3′. HSP60: forward primer, 5′-GGATTCGGAGACAACAGGAA-3′; reverse primer, 5′-CTTTGAGGAGCATCGTGTCA-3′. HSC70: forward primer, 5′-TGCTGGAGACACTCACTTGG-3′; reverse primer, 5′-GCCTGAGTGCTGGAGGATAG-3′. Thermal cycling condi-

Table 2
List of GSPs for RACE and their sequences

Name	Primer sequence (5′–3′)
GSP1 for HSP27	GGAATCTGGCGTCTGCTTGATCTCAGACAT
GSP2 for HSP27	CTTTGGGATGCCACACTTCTCCGAGGAGAT
GSP1 for HSP47	GTTCTGGACATGGCACTGGCCCATAAGATG
GSP2 for HSP47	CGAGATGTTTCTGCAGGTTGTGGCTGACCT
GSP1 for HSP60	CTCCGGGATTCGGAGACAACAGGAAAAACC
GSP2 for HSP60	CTGTGATCTCATTCGCACGCTTCTCAATGG

```
                                                                1 ga
  3 gcacttgccagaaaactttcagagcaagaaacttcagcc**atg**gcagagagacgcatcccc
                                                      M  A  E  R  R  I  P
 63 ttcaccttcatgcacggccaatcctgggatcctttccgtgactggtcccagggcagccgg
    F  T  F  M  H  G  Q  S  W  D  P  F  R  D  W  S  Q  G  S  R
123 ctcttcgatcagacctttgggatgccacacttctccgaggagatgcccacatttcccagc
    L  F  D  Q  T  F  G  M  P  H  F  S  E  E  M  P  T  F  P  S
183 acacactggcctggatactttcggccctatggatttccagagatggcctctttaatgcag
    T  H  W  P  G  Y  F  R  P  Y  G  F  P  E  M  A  S  L  M  Q
243 agcccagtggctcagatgcccatgtcgccgcccgcctccatgatgcacccccccgacctac
    S  P  V  A  Q  M  P  M  S  P  P  A  S  M  M  H  P  P  T  Y
303 agccgggctctttcccgacagatgagctcgggaatgtctgagatcaagcagacgccagat
    S  R  A  L  S  R  Q  M  S  S  G  M  S  E  I  K  Q  T  P  D
363 tcctggaagatcagcctggatgtcaatcacttcgccccagaggagctgatggtgaagacc
    S  W  K  I  S  L  D  V  N  H  F  A  P  E  E  L  M  V  K  T
423 aaagatggggtggtggagatcaccggcaaacatgaggagcggaaggatgaacatggcttt
    K  D  G  V  V  E  I  T  G  K  H  E  E  R  K  D  E  H  G  F
483 gtgtccagatgtttcaccaggaaatacactctgccctctggtgtcgactcggagaagatc
    V  S  R  C  F  T  R  K  Y  T  L  P  S  G  V  D  S  E  K  I
543 acctcgtctctgtctcctgaggggtcctgaccattgaagccactctgcccaaacctgcc
    T  S  S  L  S  P  E  G  V  L  T  I  E  A  T  L  P  K  P  A
603 atccagggccctgaagtcaacatccctatcaacacaggcagcgcagtgactgccagcagt
    I  Q  G  P  E  V  N  I  P  I  N  T  G  S  A  V  T  A  S  S
663 acaaagaaaccc**tga**gcgctacaacacacacacacacacacacacacacacacacaca
    T  K  K  P  *
723 caggcagatcactgtgctactttgagtttgttttcttcagcatgaatgcaaatgttgata
783 cttctattctttgtaaacagcaggtaggagaagaggaattgccttattctataaccatct
843 catttttacatcactctttttcatcattgtctcttctcttatcatcgtcttctcttctt
903 cttgtagtaaaaacattgactgtgtacacaccatgctctataaacatgctcatattacac
963 cctaaataaataaataaataaagattaaatataaatcatatttgcataagacatcagctg
1023 ttttatgtgcatatatatagtgcatgcacattatttcagacagttaaacattttacccca
1083 ttattgaaatatgtagtattacatattaagaacataataaattataattgtgcatatatc
1143 ctgggaacatttgtttatatacacttttttaaaatactgtctggagcgattgattgtat
1203 tcaatgtgactactacaagacgtgaacataataaaataatgtaaaaatgcaagtgttcct
1263 aaaatgtggaatgaattatgacaatgacatttctttaggaggttcccaaaacagtagttt
1323 taccatttactggtaaaattatgttttttttttataatatttattcatcaaaatgtaat
1383 acattgctctgcctgaatttttttttttctgctattgtatgtcaccttgttcatgtttg
1443 ctgttagtgaacgttgacaatactgtagtattttagccattgttttaggctacaataatg
1503 tagctttttaattccattcatatatgaagctgaagaaactgaaaaggaaggatagcctg
1563 tttccttttgaaatgagaaaaattatattcttgactttatcatacaaatagattccacca
1623 gacatcgccatgctgtttaactgcaggaggcaccacaacatggtttcctcacatcagtcg
1683 tctcagccagaagaacagcagggaatgtcttcagattggtatctttgggttttgtgaggc
1743 attgcatactgttttaaatgactgtaattaaagctttcacaatcgtgagctgtatttctg
1803 aatatccacatggtggagttgggaataaacatcttcaggcagtctggattatctgctgt 1861
```

Fig. 2. Nucleotide and deduced amino acid sequence for goldfish HSP27. Initiation and stop codons are present in bold. The sequence has been deposited in GenBank (accession no. DQ872651).

tions were as follows: 95 °C for 15 min, and 45 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 for 30 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, as displayed by a single peak (data not shown). Subsequently, fold differences in HSP expression (compared to goldfish in Huairou) were calculated using the $2^{-\mathrm{DeltaDeltaCT}}$ method (Livak and Schmittgen, 2001).

2.9. Protein extraction, gel electrophoresis and immunoblotting

Protein was extracted from the hepatic tissue of goldfish from Gaobeidian Lake and the reference site using Trizol (Invitrogen, USA). Protein was analyzed using a 4% (stacking) and 10% (separating) polyacrylamide gel, and transferred to a nitrocellulose membrane (Amresco, USA). HSP70 and actin were detected with mouse anti-HSP70 monoclonal antibody (1:1000; StressGen, Canada) and goat anti-actin polyclonal antibody (1:1000; Santa Cruz, USA), respectively. The signals were visualized by 3, 3′-Diaminobenzidine (DAB) detection and the protein bands were scanned and quantitated with Bandleader software. Anti-actin antibody served as the loading control.

2.10. Statistical analysis

All values are expressed as mean ± SE of 3 replicates in one representative experiment. All experiments were performed three times to confirm the results. A two-tailed Student's *t* test was used to determine the statistical difference between the two groups and $P<0.05$ was considered as significant. All the analyses were carried out using SPSS software (Version 13.0).

3. Results

3.1. The partial cDNA sequences of HSPs in goldfish

Single fragments of 226, 541, 539, 386, 457, 1036, and 189 bases of nucleotide sequences for HSP27, HSP30, HSP47, HSP60, HSP70, HSC70, and HSP90 beta, respectively were determined. The sequence data were deposited in GenBank with following the accession numbers: DQ872644, DQ872645, DQ872646, DQ872647, DQ872648, DQ872649, and DQ872650. The sequences of all fragments were conserved compared with their corresponding genes from zebrafish and other vertebrates (data not shown).

3.2. The full-length sequence of HSP27, HSP47 and HSP60 in goldfish

1861, 1906, and 1728 nt full-length cDNA of HSP27, HSP47, and HSP60 (excluding the RACE primers), respectively were identified using RACE-PCR. The sequence for HSP27 was deposited in GenBank with accession no. DQ872651. The

```
Rattus          MTERRVPFSLLRSPSWEPFRDWYPAHSRLFDQAFGVPRFPDEWSQWFSSAGWPGYVRPLP 60
Mus             MTERRVPFSLLRTPSWGPFRDWYPAHSRLFDQAFGVPRLPDEWSQWFSAAGWPGYVRPLP 60
Homo            MTERRVPFSLLRGPSWDPFRDWYP-HSRLFDQAFGLPRLPEEWSQWLGGSSWPGYVRPLP 59
Canis           MTERRVPFSLLRSPSWDPFRDWYPAHSRLFDQAFGLPRLPEEWAQWFGHSGWPGYVRPIP 60
Carassius        MAERRIPFTFMHGQSWDPFRDWSQ-GSRLFDQTFGMPHFSEEMPT-FPSTHWPGYFRPYG 58
Danio           MAERRIPFSFMRSPSWDPFRDWYQ-GSRLFDQSFGMPALSEEMLT-FPSTHWPGYMRPFG 58
Oncorhynchus     MTERRIPFSLLRTPSWDPYRDWYQ-GNRLFDQSFGMPAHAEGLPS-FSSTHWPGYMRPAL 58
                *:***:**::::  ** *:***    .*:***:**:*  .:     :  : ****.**

Rattus           -------------AATAEGP---------AAVTLARP----------FSRALNRQLSSGV 88
Mus              -------------AATAEG----------LAVTLAAPA---------FSRALNRQLSSGV 88
Homo             -------------PAAI------------ESPAVAAPA---------YSRALSRQLSSGV 85
Canis            -------------PAVEGPA---------AAAAAAAPA---------YSRALSRQLSSGV 89
Carassius         ---------FPEMASLMQSPVAQMP-MSPPASMMHPP---------TYSRALSRQMSSGM 99
Danio            ---------HPDFAALMQGP--------PVMPPMMTP---------SYGRALSRQLSSGM 92
Oncorhynchus       GHDLSSAGFMPSMMPHQQSAMMPLVPMMPQAPMMVQAPMMAQAGAASYSRALSRQLSTGM 118
                             .                 .        :.***.**:*:*:

Rattus           SEIRQTADRWRVSLDVNHFAPEELTVKTKEGVVEITGKHEERQDEHGYISRCFTRKYTLP 148
Mus              SEIRQTADRWRVSLDVNHFAPEELTVKTKEGVVEITGKHEERQDEHGYISRCFTRKYTLP 148
Homo             SEIRHTADRWRVSLDVNHFAPDELTVKTKDGVVEITGKHEERQDEHGYISRCFTRKYTLP 145
Canis            SEIRQTADRWRVSLDVNHFAPEELTVKTKDGVVEITGKHEERQDEHGYISRRLTPKYTLP 149
Carassius         SEIKQTPDSWKISLDVNHFAPEELMVKTKDGVVEITGKHEERKDEHGFVSRCFTRKYTLP 159
Danio            SEVKQTGDSWKISLDVNHFSPEELNVKTKDGVLEITGKHEERKDEHGFISRCFTRKYTLP 152
Oncorhynchus           SEIKQTQEAWKVTLDVNHFSPEELVVKTKDGVVEITGKHEERKDEHGFVSRCFTRKYTLP 178
                **:::* : *:::******:*:** ****:**:**********:****::** :* *****

Rattus           PGVDPTLVSSSLSPEGTLTVEAPLPKAVTQSAEITIPVTFEARAQIGGPE---SEQSGAK 205
Mus              PGVDPTLVSSSLSPEGTLTVEAPLPKAVTQSAEITIPVTFEARAQIGGPEAGKSEQSGAK 208
Homo             PGVDPTQVSSSLSPEGTLTVEAPMPKLATQSNEITIPVTFESRAQLGGPEAAKSDETAAK 205
Canis            PGVDPTLVSSSLSPEGTLTVEAPMPKPATQSAEITIPVTFEARAQIGGPEAGKSEQSGAK 209
Carassius         SGVDSEKITSSLSPEGVLTIEATLPKPAIQGPEVNIPINTGSAVTASSTKKP-------- 211
Danio            PGVDSEKISSCLSPEGVLTVEAPLPKPAIQAPEVNIPVNKTTVSTTK------------- 199
Oncorhynchus      PMADAEKVTSTLSPEGVLTVETPLNRQAIKAAEISIPVAMGSSKTKP------------- 225
                . .*.  ::* *****.**:*:.: : .  . *:.**:   :
```

Fig. 3. Alignment of HSP27 orthologs using the algorithm ClustalW. The numbers on the left indicate the amino acid positions of each protein. The "*" and ":" indicated constitute and semi-constitute amino acids. Dashes represent gaps introduced to optimize alignment. The consensus phosphorylation sites for MAPKAP kinase-2 (–RXXS–) in *Homo sapiens* HSP27 are indicated in the hatched box. *Rattus: Rattus norvegicus* (GenBank accession no. NP_114176); *Homo*: *Homo sapiens* (GenBank accession no. NP_001531); *Danio*: *Danio rerio* (GenBank accession no. NP_001008615); *Carassius*: *Carassius auratus* (GenBank accession no. ABI26639); Rainbow: *Rainbow trout* (GenBank accession no. AAZ14862); *Mus*: *Mus musculus* (GenBank accession no. NP_038588). *Canis*: *Canis familiaris* (GenBank accession no. P42929).

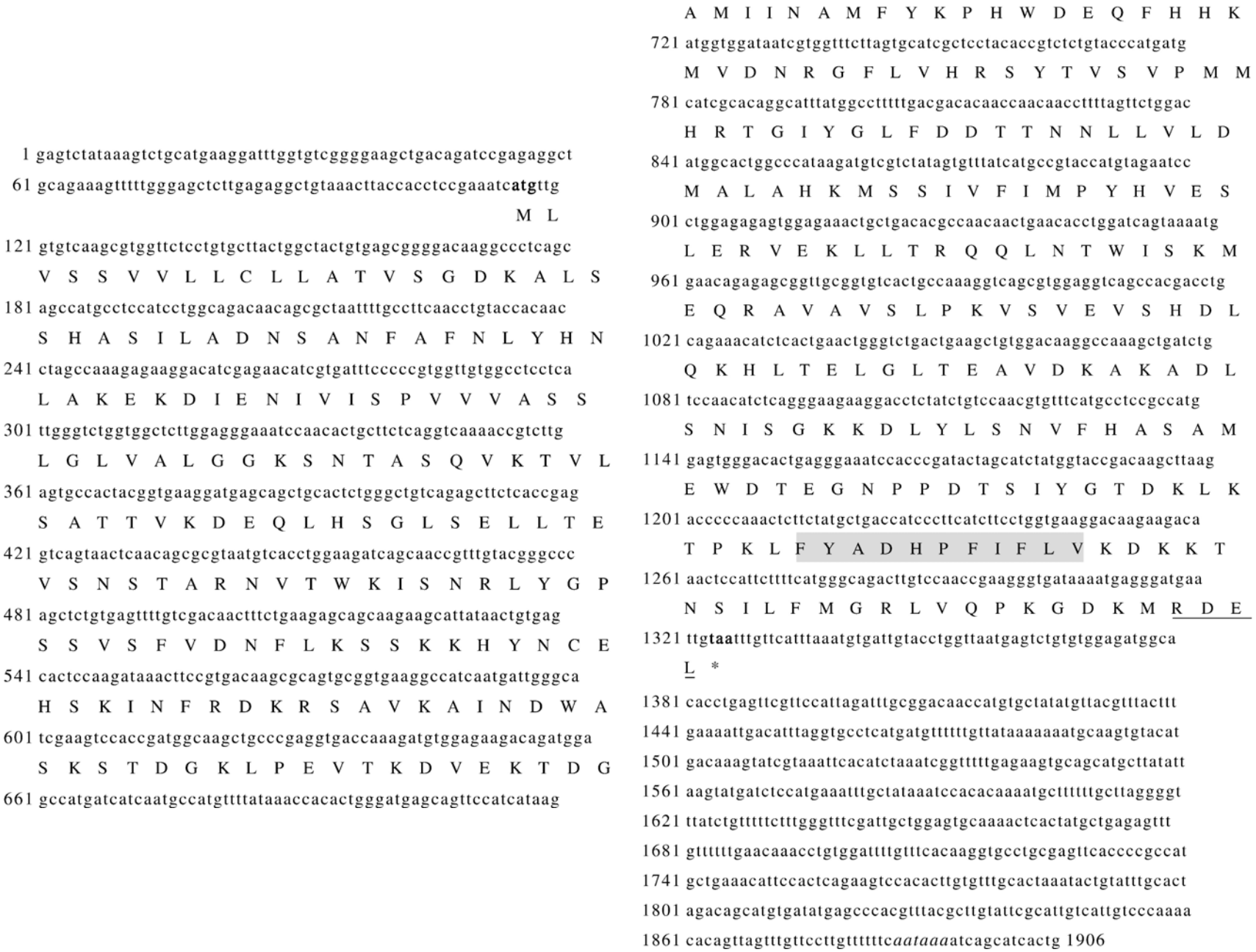

Fig. 4. Nucleic acid and derived amino acid sequence of goldfish HSP47. The sequence has been deposited in GenBank (accession no. DQ872652). Initiation and termination codons are in bold. The polyadenylation signal at nt 1888 to 1894 is in italics. The RDEL endoplasmic reticulum targeting sequence (aa 400 to 403) is underlined, and the serpin signature at aa 367 to 377 is shown in a hatched box.

HSP27 mRNA produced a peptide sequence of 168 amino acids in length putatively, with theoretical pI 6.38 and a mass of 23.76 kDa (Fig. 2). The deduced amino acid sequences of HSP27 in goldfish had 54% to 77% similarities to HSP27 in other investigated vertebrates (Fig. 3). HSP47 cDNA in goldfish (GenBank accession no. DQ872652) contained an ORF of 1212 nt putatively, encoding a protein of 403 amino acids. A polyadenylation signal, AATAAA, was found at nt 1888 to 1894, located upstream of the poly (A) tract (Fig. 4). The full-length cDNA of goldfish HSP60 (GenBank accession no. DQ872653) included an uninterrupted reading frame of 1728 nt, along with 123 nt of a 5′ untranslated region (UTR) and a 3′ UTR of 721 nt (RACE primer sequence not included) (Fig. 5). The putative 575 amino acids product had a theoretical pI of 5.40 and a mass of 61.05 kDa.

3.3. Spatial expression of HSP27, HSP47 and HSP60 transcripts in Huairou Reservoir

Each cDNA sample from goldfish brain, heart, liver, gill, skeletal muscle and kidney was amplified for 30 cycles with HSP27, HSP47 and HSP60 primers (described in Section 2.3

Fig. 5. Nucleotide and deduced amino acid sequence for goldfish HSP60. Initiation and stop codons are in bold. The sequence has been deposited in GenBank (accession no. DQ872653). The conserved ATP/ADP (aa 76 to 82) and Mg^{2+} binding (aa 109 to 119) sites are indicated in italics and underlined respectively, and substrate binding sites (aa 215 to 227) in a hatched box.

```
    V K A P G F G D N R K N Q L Q D M A I S
1081 accggaggcacggtgtttggtgatgaggctgtgggtctggccattgaggacatccaggca
    T G G T V F G D E A V G L A I E D I Q A
1141 catgacttcggcagggtcggcgaggtcattgtgaccaaggatgacacgatgctcctcaaa
    H D F G R V G E V I V T K D D T M L L K
1201 ggccgtggtgatccagcagccattgagaagcgtgcgaatgagatcacagaacagctggag
    G R G D P A A I E K R A N E I T E Q L E
1261 agcaccaacagtgactacgagaaggagaaactcaacgagcgtctggccaagctctctgat
    S T N S D Y E K E K L N E R L A K L S D
1321 ggagtggctgtgattaaggttggaggaacaagtgacgttgaagtgaatgagaagaaggac
    G V A V I K V G G T S D V E V N E K K D
1381 cgtgtcactgatgcgctgaacgccactcgagccgctgtggagggggggaatcgttcttgga
    R V T D A L N A T R A A V E G G I V L G
1441 ggaggatgtgccctgctgcgctgcatcccagccctggacaacatcaagccagccaataat
    G G C A L L R C I P A L D N I K P A N N
1501 gatcaaaagatcggtatcgaaattattcgcagtgcgcttcgtattcctgcaatgaccatt
    D Q K I G I E I I R S A L R I P A M T I
1561 gccaagaatgcaggagttgacggctctctggtggtggagaagatcttgcagagcgctcca
    A K N A G V D G S L V V E K I L Q S A P
1621 gagattggatatgatgctatgaatggagaatatgtaaacatggtcgaaagaggcattatt
    E I G Y D A M N G E Y V N M V E R G I I
1681 gaccccacaaaggtcgtgaggactgcattgctagatgctgcaggtgttgcatctctgctg
    D P T K V V R T A L L D A A G V A S L L
1741 gctactgctgaagctgtagtcactgagataccaaaggaggagaaggacacgccagctgga
    A T A E A V V T E I P K E E K D T P A G
1801 ggaatgggtggaatgggaggcatgggaggtatgggtggcatgggattctaaactgatctg
    G M G G M G G M G G M G G M G F *
1861 cactgactttagtgaaagggttgagggcaggggacatgatttgcccctccctttcaactt
1921 ggaaaaaccttctgaaattgattgaaggtggtctgatcttggaccaaaataaatggacct
1981 tccagtctcccatccttctactactatgtccaatatcatctctctactcatggctgaaga
2041 tgacaccctactgctttaaagacaagttcttgataatgtgactagagagagtgtctgcct
2101 gttctgtaacattgtgttcaccaacatctaatgggaaagtgcatttaacattgcatatta
2161 tcttgctattgtatttatgaatgtcattgtgggcggcattgtagttttgaaaaagccag
2221 acgttcaggttcaagtttgtcttcataagcagggaagtagtaagaacttttgaccagtta
2281 tatctaaaaaaataaataataataattgcacttccacgatcagtaacacttttttttttt
2341 ttttgtacaatgtttgtttcatatgaaaataaacccctcatttgaggattactcaaatgt
2401 gaggaaaaactgtgacgaagcatgtacttgttttgccttcaattgttacatgaatttttc
2461 tcagtgactagaactacattgctgtaagcgtaaagaattgtgtgataacatttgtgttcc
2521 aggtttcatttcttaaagggatggttctcctaaaaataaaaacctgtcattt 2572
```

Fig. 5 (*continued*).

above). Parallel reactions using beta-actin specific primers were also performed for 26 cycles with the same parameters used for HSPs (Fig. 6). HSP27 transcripts were detected in all of the tissue tested, but the levels were relatively higher in heart, brain, gill, and skeletal muscle, especially the heart, relative to the expression of beta-actin specific mRNA. The higher expression

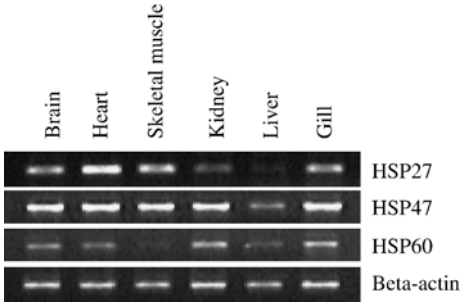

Fig. 6. RT-PCR analysis of HSP27, HSP47 and HSP60 expression at 30 cycles for various goldfish tissues. Total RNA was isolated from the brain, heart, skeletal muscle, kidney, liver and gill tissues, then reverse transcribed and amplified. The expression of beta-actin at 26 cycles was used as an internal control.

of goldfish HSP27 mRNA in the heart was in agreement with zebrafish HSP27 specific mRNA (Mao et al., 2005) in which low level expression of zebrafish HSP27 mRNA was reported for brain and gill tissues. HSP47 was abundantly expressed in all of the tissues examined except the liver. A lesser amount of HSP47 mRNA was detected in the latter. HSP60 transcripts were detected at lower levels in the heart, brain, liver, and skeletal muscle. A relatively higher expression of HSP60 mRNA was detected in the kidney and gill.

3.4. HSPs mRNA expression under the complex environment in Gaobeidian Lake

In order to select the exponential phase of amplification and so be able to compare the expression levels of hepatic HSPs in goldfish from Gaobeidian Lake and the reference site, on November 23, 2005, PCR assays with different cycle numbers (20, 25, 30, 35 and 40 cycles) were performed to view the amplification curve of all the HSPs tested using hepatic cDNA as template. Based on the amplification curves (data not shown), 33, 32, 29, 31, 31, 22 and 27 cycles of PCR for HSP27, HSP30, HSP47, HSP60, HSP70, HSC70 and HSP90 beta, respectively, were selected as the optimal cycle numbers for measuring the levels of mRNA expression. cDNA performed in the absence of reverse transcriptase showed no visual amplifications (data not shown). Subsequently, the amplification results showed that HSP30 and HSP70 had statistically higher levels of mRNA expression in goldfish liver from Gaobeidian Lake than those from the reference site (Fig. 7).

Real-time quantitative PCRs were performed on goldfish hepatic samples collected from the two field sites on May 16, 2006. Beta-actin was used as an endogenous control. The results indicated that mRNA expression of members of the HSP family, including HSP27, HSP30, and HSP90 beta were higher in goldfish from Gaobeidian Lake than those from the reference site, especially HSP30, which showed a 32 fold increase (Table 3).

3.5. HSP70 protein expression under complex environment in Gaobeidian Lake

Confirmatory Western blots were also performed on goldfish hepatic samples obtained on May 16, 2006. No detectable change of HSP70 was found between fish obtained from Gaobeidian Lake and the reference site after they were normalized against beta-actin (Fig. 8).

4. Discussion

Goldfish, a eurythermal fish, which can survive over a wide temperature range, is an ideal organism in which to study heat shock response. In the current study, cDNA sequences encoding members of HSP family were cloned from goldfish.

The size of sHSPs range from 12 to 42 kDa and they can form large multimeric structures and perform a wide range of cellular functions. One major characteristic of this protein family is the presence of a sequence of about 100 residues (which is homologous to alpha-crystallin from the vertebrate eye lens) called the alpha-crystallin or small heat shock protein domain, flanked by an N-terminal region and a C-terminal extension (Kim et al., 1998). While the N-terminal region and the C-terminal extension are variable in length and sequence, the highest degree of amino acid similarity is found within the alpha-crystallin domain. Most sHSPs assemble into large polymeric units, ranging from 100 kDa to 1 MDa, for efficient chaperone activity. The putative amino acid sequence of the goldfish HSP27 which we cloned has an alpha-crystallin domain of 101 to 183 amino acid residues, and is a member of the small heat shock family.

The deduced amino acid sequence of goldfish HSP27 revealed that it contains two phosphorylation sites for MAPKAP kinase-2 (–RXXS–), and the consensus phosphorylation site at position aa 12–15 in human HSP27 (Landry et al., 1992; Mehlen and Arrigo, 1994) had disappeared due to a substitution at amino acid residue 12. The phosphorylation of HSP27 was involved in the stabilization of the actin filament as well as in promoting actin polymerization and stress-fibre formation (Guay et al., 1997; Landry and Huot, 1999).

A BLASTN search revealed another HSP27 sequence from goldfish (GenBank accession no. AB239443), with a putative ORF of 612 nt. There were few substitutions and two deletions that distinguished the two goldfish HSP27 sequences. Overall, the two shared 88% identity in nucleotide sequence and 87% identity in the deduced amino acid sequences. Presumably, the two clones were derived from gene duplication, one genetic basis for the origin of a multigene family. AB239443 had a shorter C-terminal tail compared with DQ872651. Studies have shown that the flexible C-terminal tail found on almost all sHSPs allowed them to interact with cellular proteins and played a key regulatory role in the maintenance of target protein solubility (Fernando et al., 2002). Thus, the two paralogs may play diverse roles in chaperoning of the misfolding proteins under stress conditions.

A member of the 47 kDa HSP family has been reported in eukaryotic organisms (Nagata, 1996). The deduced translation product of the goldfish HSP47 cDNA is 76% identical to zebrafish (GenBank accession no. AAH71301), 67% identical to rat (GenBank accession no. AAH86529), and 65% identical to human HSP47 (GenBank accession no. AAH36298) proteins. Mammalian HSP47, located in the endoplasmic reticulum

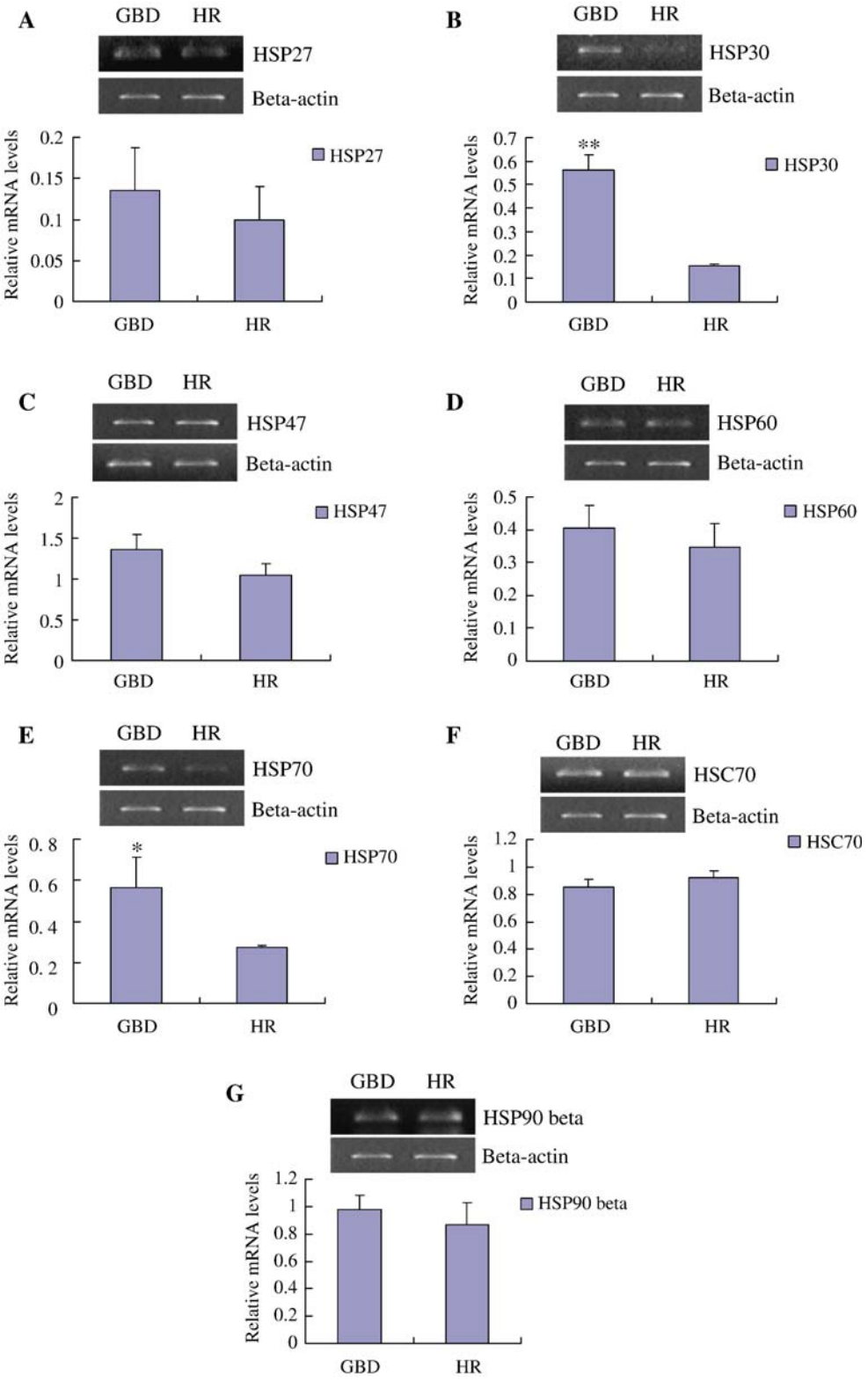

Fig. 7. Effect of environmental stress on expression of HSPs shown by RT-PCR. Hepatic tissues of goldfish obtained from Gaobeidian Lake (GBD) and Huairou Reservoir (HR) on November 23, 2005 were excised. Total RNA was isolated, reverse-transcripted, and subjected to PCR assay. The amplification cycles for HSP27 (A), HSP30 (B), HSP47 (C), HSP60 (D), HSP70 (E), HSC70 (F), and HSP90 beta (G) were 33, 32, 29, 31, 31, 22, and 27, respectively. RT-PCR products were subjected to electrophoresis on a 1.2% agarose gel. Band densities were normalized for beta-actin (26 cycles) levels. Each value represents the mean ± SE of 3 replicates in one representative RT-PCR. The representative ethidium bromide stained gels are also shown. The "*" and "**" denote statistical significance ($P < 0.05$ and $P < 0.01$ respectively).

Table 3
Environmental stress effect on expression of HSPs shown by real-time PCR

Gene	DeltaC$_T$ (GBD)	DeltaC$_T$ (HR)	$2^{-\text{Delta Delta CT}}$
HSP27	7.06±0.57**	7.52±0.71	5.50 (3.71–8.40)
HSP30	6.47±0.90**	11.49±1.46	32.45 (17.27–60.55)
HSP47	9.19±1.15	10.94±2.70	3.37 (1.53–7.46)
HSP60	15.74±1.34	17.98±1.63	4.72 (1.87–12.04)
HSP70	12.89±3.70	9.74±0.52	0.11 ($8.68E^{-3}$–1.45)
HSC70	−1.59±0.38	−1.04±0.77	1.46 (1.23–1.91)
HSP90 beta	−1.9±0.5 *	−0.52±0.38	2.60 (1.73–3.96)

Delta C$_T$: Avg. target C$_T$-Avg. beta-actin C$_T$.
GBD: Gaobeidian Lake; HR: Huairou Reservoir.
Delta Delta C$_T$: Avg. Delta C$_T$ (GBD)-Avg. Delta C$_T$ (HR).
The "*" and "**" denote statistical significance ($P<0.05$ and $P<0.01$, respectively).

(ER) in cells, is a collagen-specific molecular chaperone (Nagata and Hosokawa, 1996; Nagata, 2003). As with HSP47 in higher vertebrates, the goldfish HSP47 protein also contains an RDEL endoplasmic reticulum targeting sequence (aa 400–403). In addition, HSP47 in higher vertebrates shows a weak but significant sequence similarity with the serine proteinase inhibitor (serpin) family of proteins (Hirayoshi et al., 1991), and the serpin signature at aa 367–377 was also found in goldfish HSP47.

HSP60 is a well-characterized chaperone localized mainly in the mitochondria of eukaryotic cells (Cheng et al., 1989; Martin et al., 1992; Soltys and Gupta, 1996). HSP60 can elicit a potent proinflammatory response in cells of the innate immune system and therefore has been hypothesized as a danger signal of stressed or damaged cells (Ohashi et al., 2000). The amino acid sequences of the chaperonin proteins are highly conserved in both prokaryotes and eukaryotes (Gupta, 1995; Brocchieri and Karlin, 2000; Martin et al., 2002). Sequence analysis and alignment were carried out using clustalW with HSP60s from goldfish, mouse, rat, human, zebrafish and other eukaryotes (data not shown). The overall sequence identity of goldfish HSP60 is 85% homologues to human HSP60 (GenBank accession no. AAH73746); 86% to rat HSP60 (GenBank accession no. AAC53362), and 94% to zebrafish HSP60 (GenBank accession no. AAH68415). The sequence analyses revealed a number of HSP60 protein domains that are conserved across phyla including the ATP/ADP (DGVTVAK) and Mg^{2+}binding sites (AGDGTTTATVL) and substrate binding sites (EGMKFDRGYISPY) (Fenton et al., 1994). Interestingly, recent study of zebrafish showed that a V324E missense mutation in the HSP60 gene, which reduced HSP60 function in binding and refolding denatured proteins, leads to an early fin regeneration defect, indicating that HSP60 may play a vital role in the formation and maintenance of regenerating tissue (Makino et al., 2005).

Studies in laboratory conditions have well documented that the expressions of HSPs are up-regulated when an organism is exposed to acute stress conditions, such as acute temperature shift, but that this may not be easily related to natural conditions. Hofmann and his colleagues (2000) examined the occurrence of the heat shock response in a highly cold-adapted, stenothermal Antarctic teleost fish, *Trematomus bernacchii*, and the result showed that the fish when subjected to a heat stress yielded no evidence for synthesis of any size class of HSP family, so it remains to be established how chronic adaptation modulates HSPs expression in the field. In our present study, significant up-regulation in HSP30 and HSP70 transcripts was exhibited in goldfish collected from Gaobeidian Lake in winter, while up-regulation in HSP27 and HSP90 beta transcript, as well as in HSP30, was observed in goldfish collected from Gaobeidian Lake on the day of collection in summer. The different expression of HSPs is due probably to samples being collected at different seasons and/or using different detection methods. It is noteworthy that HSP30 was evidently expressed in Gaobeidian Lake under complex stress conditions. In contrast, it has a very weak expression in goldfish liver from the reference site, indicating that HSP30 may serve as a good biomarker for this kind of complex stress exposure.

HSP70 is among the most widely studied heat shock proteins in vertebrates and is believed to be a very good biomarker for heat stress. The HSP70 antibodies used in immunoblotting could recognize both constitutive and inducible members of the 70 kDa HSP family. A single band of about 70 kDa was present at detectable levels in the hepatic tissues of fish specimens from both Gaobeidian Lake and the reference site, although the group of goldfish collected from the lake in November, 2005 displayed an increase in HSP70 mRNA accumulation in liver tissue, and its up-regulation was not detected statistically at either transcriptional or translational levels in samples collected on May 16, 2006.

The complex environmental conditions in Gaobeidian Lake had no effect on the expression of HSC70, the constitute expression form of heat shock protein 70. Thus the consistent nature of HSC70 expression under different environmental conditions indicated that HSC70 could be used as an internal control in normalization of the expression of target genes (Airaksinen et al., 2003a,b; Ojima et al., 2005). In addition, the differential expression was not detected in hepatic HSP47, and HSP60 mRNA suffered complex stress in Gaobeidian Lake. This result differs from that obtained under the acute stress conditions reported earlier (Murtha and Keller, 2003; Choresh et al., 2001).

The up-regulation of the genes of members of HSP families in Gaobeidian Lake strongly indicated that stress response is indeed activated in goldfish residing in this environment. Transcription of mammalian HSP genes requires the activation

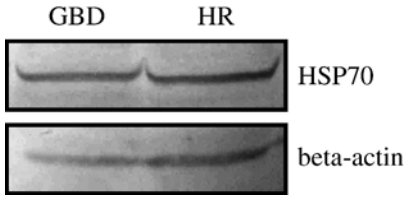

Fig. 8. Protein expression of HSP70 shown by western blot analysis. Protein in hepatic tissues of goldfish from Gaobeidian Lake (GBD) and Huairou Reservoir (HR) was extracted, and analyzed using a 4% (stacking) and 10% (separating) polyacrylamide gel. The signals were visualized by DAB detection. Anti-actin antibody served as the loading control.

and translocation of heat shock transcription factors (HSFs) to the nucleus, where the heat shock responding elements located within the HSP gene promoter region are recognized (Santoro, 2000; Morimoto, 1998). It has been shown that in fish also the HSE is occupied by HSFs upon HSP70 induction (Airaksinen et al., 1998). Although the expressions of some HSPs were up-regulated under the complex environmental conditions in Gaobeidian Lake, their transcriptional activities were not unified. The expression of these genes may vary from HSP family member to member, and with toxicant concentration and/or thermal conditions due to diverse HSF binding activity and other transcriptional modification mechanisms. In summary, the expression of stress response proteins was not consistent in responding to differential stressors. The complexity and variability of the environment tends to confound the establishment of links between stressor and the responding HSP family members. However, our results do highlight the need for more research to determine whether HSP30 can be used as a biomarker of the complex environmental exposure.

Acknowledgements

This work was funded by the Innovation Project of the Chinese Academy of Sciences (No: SCX2-SW-128 to Dai) and 973 Program (2006CB403306). We would like to thank Dr. Aichun Dong, (University of Northern Colorado, USA) and Dr. Jingxiang Bai (Mount Sinai School of Medicine, USA) for their helpful comments on the manuscript. We also thank Professor I.J. Hodgkiss for polishing the English.

References

Airaksinen, S., Rabergh, C.M., Sistonen, L., Nikinmaa, M., 1998. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. J. Exp. Biol. 201, 2543–2551.

Airaksinen, S., Jokilehto, T., Rabergh, C.M., Nikinmaa, M., 2003a. Heat- and cold-inducible regulation of HSP70 expression in zebrafish ZF4 cells. Comp. Biochem. Physiol. B 136, 275–282.

Airaksinen, S., Rabergh, C.M., Lahti, A., Kaatrasalo, A., Sistonen, L., Nikinmaa, M., 2003b. Stressor-dependent regulation of the heat shock response in zebrafish, *Danio rerio*. Comp. Biochem. Physiol. A 134, 839–846.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173–183.

Beckmann, R.P., Mizzen, L.E., Welch, W.J., 1990. Interaction of Hsp70 with newly synthesized proteins: implications for protein folding and assembly. Science 248, 850–854.

Brocchieri, L., Karlin, S., 2000. Conservation among HSP60 sequences in relation to structure, function, and evolution. Protein Sci. 9, 476–486.

Broome, C.S., Kayani, A.C., Palomero, J., Dillmann, W.H., Mestril, R., Jackson, M.J., McArdle, A., 2006. Effect of lifelong overexpression of HSP70 in skeletal muscle on age-related oxidative stress and adaptation after nondamaging contractile activity. FASEB J. 20, 1549–1551.

Cheng, M.Y., Hartl, F.U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L., Horwich, A.L., 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature 337, 620–625.

Chiang, H.L., Terlecky, S.R., Plant, C.P., Dice, J.F., 1989. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246, 382–385.

Choresh, O., Ron, E., Loya, Y., 2001. The 60-kDa heat shock protein (HSP60) of the sea anemone *Anemonia viridis*: a potential early warning system for environmental changes. Mar. Biotechnol. (NY) 3, 501–508.

Fenton, W.A., Kashi, Y., Furtak, K., Horwich, A.L., 1994. Residues in chaperonin GroEL required for polypeptide binding and release. Nature 371, 614–619.

Fernando, P., Abdulle, R., Mohindra, A., Guillemette, J.G., Heikkila, J.J., 2002. Mutation or deletion of the C-terminal tail affects the function and structure of *Xenopus laevis* small heat shock protein, hsp30. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 133, 95–103.

Gamperl, A.K., Vijayan, M.M., Pereira, C., Farrell, A.P., 1998. Beta-receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. Am. J. Physiol. 274, R428–R436.

Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J.N., Huot, J., Landry, J., 1997. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J. Cell Sci. 110, 357–368.

Gupta, R.S., 1995. Evolution of the chaperonin families (Hsp60, Hsp10 and Tcp-1) of proteins and the origin of eukaryotic cells. Mol. Microbiol. 15, 1–11.

Hallare, A.V., Kohler, H.R., Triebskorn, R., 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56, 659–666.

Hallare, A.V., Kosmehl, T., Schulze, T., Hollert, H., Kohler, H.R., Triebskorn, R., 2005. Assessing contamination levels of Laguna Lake sediments (Philippines) using a contact assay with zebrafish (*Danio rerio*) embryos. Sci. Total Environ. 347, 254–271.

Hirayoshi, K., Kudo, H., Takechi, H., Nakai, A., Iwamatsu, A., Yamada, K.M., Nagata, K., 1991. HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. Mol. Cell. Biol. 11, 4036–4044.

Hofmann, G.E., Buckley, B.A., Airaksinen, S., Keen, J.E., Somero, G.N., 2000. Heat-shock protein expression is absent in the antarctic fish *Trematomus bernacchii* (family Nototheniidae). J. Exp. Biol. 203, 2331–2339.

Kagawa, N., 2004. A drastic reduction in the basal level of heat-shock protein 90 in the brain of goldfish (*Carassius auratus*) after administration of geldanamycin. Zoolog. Sci. 21, 1085–1089.

Kim, K.K., Kim, R., Kim, S.H., 1998. Crystal structure of a small heat-shock protein. Nature 394, 595–599.

Kondo, H., Harano, R., Nakaya, M., Watabe, S., 2004. Characterization of goldfish heat shock protein-30 induced upon severe heat shock in cultured cells. Cell Stress Chaperones 9, 350–358.

Landry, J., Huot, J., 1999. Regulation of actin dynamics by stress-activated protein kinase 2 (SAPK2)-dependent phosphorylation of heat-shock protein of 27 kDa (Hsp27). Biochem. Soc. Symp. 64, 79–89.

Landry, J., Lambert, H., Zhou, M., Lavoie, J.N., Hickey, E., Weber, L.A., Anderson, C.W., 1992. Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. J. Biol. Chem. 267, 794–803.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (−Delta Delta C (T)) method. Methods 25, 402–408.

Makino, S., Whitehead, G.G., Lien, C.L., Kim, S., Jhawar, P., Kono, A., Kawata, Y., Keating, M.T., 2005. Heat-shock protein 60 is required for blastema formation and maintenance during regeneration. Proc. Natl. Acad. Sci. U. S. A. 102, 14599–14604.

Mao, L., Bryantsev, A.L., Chechenova, M.B., Shelden, E.A., 2005. Cloning, characterization, and heat stress-induced redistribution of a protein homologous to human hsp27 in the zebrafish *Danio rerio*. Exp. Cell Res. 306, 230–241.

Martin, J., Horwich, A.L., Hartl, F.U., 1992. Prevention of protein denaturation under heat stress by the chaperonin Hsp60. Science 258, 995–998.

Martin, C.C., Tsang, C.H., Beiko, R.G., Krone, P.H., 2002. Expression and genomic organization of the zebrafish chaperonin gene complex. Genome 45, 804–811.

Mayr, M., Kiechl, S., Willeit, J., Wick, G., Xu, Q., 2000. Infections, immunity, and atherosclerosis: associations of antibodies to *Chlamydia pneumoniae*,

Helicobacter pylori, and cytomegalovirus with immune reactions to heat-shock protein 60 and carotid or femoral atherosclerosis. Circulation 102, 833–839.

Mehlen, P., Arrigo, A.P., 1994. The serum-induced phosphorylation of mammalian hsp27 correlates with changes in its intracellular localization and levels of oligomerization. Eur. J. Biochem. 221, 327–334.

Misra, S., Zafarullah, M., Price-Haughey, J., Gedamu, L., 1989. Analysis of stress-induced gene expression in fish cell lines exposed to heavy meals and heat shock. Biochim. Biophys. Acta 1007, 325–333.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12, 3788–3796.

Murtha, J.M., Keller, E.T., 2003. Characterization of the heat shock response in mature zebrafish (*Danio rerio*). Exp. Gerontol. 38, 683–691.

Nagata, K., 1996. Hsp47: a collagen-specific molecular chaperone. Trends Biochem. Sci. 21, 23–26.

Nagata, K., 2003. HSP47 as a collagen-specific molecular chaperone: function and expression in normal mouse development. Semin. Cell Dev. Biol. 14, 275–282.

Nagata, K., Hosokawa, N., 1996. Regulation and function of collagen-specific molecular chaperone, HSP47. Cell Struct. Funct. 21, 425–430.

Ohashi, K., Burkart, V., Flohe, S., Kolb, H., 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J. Immunol. 164, 558–561.

Ojima, N., Yamashita, M., Watabe, S., 2005. Comparative expression analysis of two paralogous Hsp70s in rainbow trout cells exposed to heat stress. Biochim. Biophys. Acta. 1681, 99–106.

Parsell, D.A., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27, 437–496.

Santoro, M.G., 2000. Heat shock factors and the control of the stress response. Biochem. Pharmacol. 59, 55–63.

Soltys, B.J., Gupta, R.S., 1996. Immunoelectron microscopic localization of the 60-kDa heat shock chaperonin protein (Hsp60) in mammalian cells. Exp. Cell Res. 222, 16–27.

Wagner, M., Hermanns, I., Bittinger, F., Kirkpatrick, C.J., 1999. Induction of stress proteins in human endothelial cells by heavy metal ions and heat shock. Am. J. Physiol. 277, L1026–L1033.